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A Method for the Simultaneous Detection of *Vibrio cholerae* Strains and Drug-Resistant Genes in their Genome by Real-Time PCR

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Abstract—A method was developed for the simultaneous detection of *V. cholerae* strains and the presence of drug-resistant genes in their genomes by real-time PCR. Resistance to four antibiotics used for cholera treatment (tetracycline, trimethoprim, chloramphenicol and ciprofloxacin) was analyzed. The sensitivity of the panel was 1×10^3 CFU/mL for pure cultures, simulated clinical material, and environmental samples. PCR efficiency was confirmed by the analysis of 60 natural *V. cholerae* strains isolated from patients and the environment in different years. It was established that all of the studied *V. cholerae* strains of the O1 serogroup El Tor biovar isolated before 1993 did not contain the tested drug-resistant genes. At the same time, 18 toxigenic clinical strains (90%) imported within 1993–2010 were characterized by multiple drug resistance; their genomes contained trimethoprim resistance genes (*dhfrAI*) and chloramphenicol resistance genes (*floR*), and 11 strains additionally contained a tetracycline-resistant gene *tetR*. In addition, nontoxigenic clinical and aqueous *V. cholerae* strains carrying a ciprofloxacin-resistant gene, the *qnrVC* (*qnrVC1*) gene, have been detected in the last few years (Kalmykia, 2011–2013).

Keywords: drug-resistant genes, multiplex real-time PCR, *Vibrio cholerae*

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INTRODUCTION

Cholera is a serious, especially dangerous, human infectious disease that can be fatal if left untreated. The causative agents of cholera are toxigenic *Vibrio cholerae* strains of the O1 (classical and El Tor biovars) and O139 serogroups. The seventh cholera pandemic, which was caused by *V. cholerae* strains of O1 serogroup El Tor biovar, has continued from 1961 to the present. Genetically distinct strains (or atypical El Tor strains) of *V. cholerae* strains of the O1 serogroup El Tor biovar [1–4] appeared and spread throughout the 1990s. These strains, unlike the typical El Tor bacillus, which caused the onset of the current pandemic, have an increased virulence that is exhibited as more severe manifestations of the disease, and they have high mortality rates. Another important feature of the atypical El Tor strains is multiple resistance to various drugs [5–7].

The atypical El Tor strains not only circulate in cholera endemic regions in Asia, Africa, and the Caribbean countries; they can also be exported to various countries, including Russia. Since 1993, all outbreaks and isolated cases of cholera in the Russian

Federation have been caused by these strains [8]. Single atypical El Tor strains were also isolated from the open reservoirs of our country during monitoring studies. A large number of nontoxigenic *V. cholerae* strains that are capable of causing intestinal infections and contain in some cases additional pathogenic genes with different resistances to antibacterial drugs were isolated from the environment. More than 700 such strains were isolated from 2007 to 2016 [9].

Cholera treatment primarily includes rehydration therapy and, in severe cases, chemotherapy with antibacterial drugs. At the same time, drugs of the tetracycline groups, fluoroquinolones, and trimethoprim are used (the latter is included in coformulated drugs such as sulfamethoxazole/trimethoprim and cotrimoxazole). Fluoroquinolones as reserve drugs are used when strains with multiple drug resistances are detected.

Antimicrobial drugs can reduce the duration and severity of the disease, but their use leads to the emergence of resistant isolates. The problem of the emergence of drug-resistant strains of pathogenic microorganisms is one of the most important issues in modern healthcare. This problem is especially acute in the treatment of especially dangerous quarantine infections, including cholera. According to the WHO, a steady increase in resistance to AMD among pathogenic bacteria, including *V. cholerae*, was detected (WHO Global

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Abbreviations: AMD—antimicrobial drug; CFU—colony-forming unit; PCR—polymerase chain reaction; Cip—ciprofloxacin; Cm—chloramphenicol; dNTP—deoxynucleoside triphosphate(s); Tc—tetracycline; Tp—trimethoprim.

Strategy for Containment of Antimicrobial Resistance:WHO/CDS/CSR/DRS/2001.2 (http://www.who.int/drugresistance/WHO_Global_Strategy_English.pdf?ua=1)). Therefore, rapid access to reliable information about the presence of drug-resistant genes in the genome of the pathogen is required to provide effective therapy for cholera patients and to obtain information about the drug resistance of imported and isolated *V. cholerae* strains during monitoring studies.

The goal of this study was to develop a method for the simultaneous detection of *V. cholerae* strains of the O1 serogroup and to determine the presence of therapeutically important drug-resistant genes in genomes. For this, multiplex real-time PCR was used.

MATERIALS AND METHODS

Bacterial Strains and Cultivation Conditions

Sixty-eight natural *V. cholerae* strains isolated on the territory of Russia and abroad in different years were used in the study (Table 1). In addition, strains of other microorganisms stored in a lyophilized state—*Escherichia coli*, *Klebsiella pneumoniae*, *Shigella sonnei*, *Salmonella typhi*, *Salmonella typhimurium*, and *Salmonella enteritidis* (State Collection of Pathogenic Bacteria of the Russian Antiplague Scientific Research Institute “Microbe”, Saratov)—were used. The bacteria were cultivated on LB agar (Sigma, United States) at 37°C.

Determination of the Sensitivity of Microorganism Cultures to Antimicrobial Drugs

The sensitivity of cultures to AMD was determined by the disc diffusion method in accordance with Practical Policies 4.2.2495-09, “Determination of the sensitivity of dangerous infectious agents (plague, anthrax, cholera, tularemia, brucellosis, glanders, melioidosis) to antibacterial drugs.” Antibiotics produced by HiMedia Laboratories Pvt Limited (India) and Research Center for Pharmacotherapy (Russia) were used. The discs contained the following AMD amounts (per disk): chloramphenicol 30 mg, tetracycline 30 µg, trimethoprim 25 µg, ciprofloxacin 30 µg.

DNA Isolation

Purified DNA was prepared with an Axy Prep Bacterial Genomic DNA Miniprep Kit (Axygen, the United States) according to the manufacturer’s protocol. Before isolation, the bacterial suspensions were treated with a 0.01% sodium merthiolate solution (Sigma-Aldrich, United States) and heated at 56°C for 30 min.

Polymerase Chain Reaction

PCR was performed with a Rotor-Gene Q 5plex (Qiagen Inc, GMBH, Germany) amplifier with

primers and TaqMan® probes synthesized by Synthol (Russia).

RESULTS AND DISCUSSION

Determination of Toxigenicity of V. cholerae Strains Used in the Study

In the first stage of the study, all *V. cholerae* biovar El Tor strains were tested with real-time PCR for the presence of the *ctxA* gene, which encodes the toxic A-subunit of cholera toxin. The primers and probe for the fragment of the *ctxA* gene were calculated by us in advance [10]. Based on the study results, we revealed 34 of 60 *V. cholerae* biovar El Tor strains containing the *ctxA* gene, i.e. toxigenic. At the same time, 26 strains isolated from human and from the environment did not carry the *ctxA* gene and were non-toxigenic (Table 1).

Determination of the Sensitivity of V. cholerae Strains to Antimicrobial Drugs

The sensitivity of all studied strains to the most commonly drugs used for the treatment of cholera AMD (chloramphenicol, tetracycline, trimethoprim, ciprofloxacin) was later determined by the disc diffusion method. The analysis demonstrated that 20 typical strains (91%) of those isolated in 1966–1988 were sensitive to the tested AMD. Two nontoxigenic *V. cholerae* strains, M1399 and M1400, were the exception; they were isolated from the environment in Astrakhan in 1982 and proved to be resistant to ciprofloxacin (Table 1).

According to the literature, *V. cholerae* biovar El Tor strains with multiple (>3) drug resistances were registered in the 1990s [11, 12]. Indeed, according to the obtained information, the toxigenic *V. cholerae* biovar El Tor strains imported into Krasnodar, Kazan and Dagestan in 1993 were resistant to tetracycline, chloramphenicol, and trimethoprim. It is interesting that the clinical strains that were imported later, including the modern isolates, were already sensitive to tetracycline. The only exception was strain M1269 (Magnitogorsk, 1994), which was resistant to this antibiotic. Strains isolated in 1993–2010 were also resistant to trimethoprim and chloramphenicol. Two strains, M1293 and M1295 (Dagestan, 1994), were an exception; these strains were resistant to trimethoprim but sensitive to levomycetin (chloramphenicol). It should be noted that the cholera in Dagestan in 1994 was caused by *V. cholerae* biovar El Tor strains with a different sensitivity to chloramphenicol. One group of strains (M1286, M1287, M1288) was resistant to this antibiotic, while the other (M1293, M1295) was sensitive (Table 1). Perhaps this was due to the fact that the cholera in Dagestan in 1994 was caused by strains imported by pilgrims from different endemic territories [13].

Table 1. Sensitivity to antibacterial preparations and the occurrence of drug-resistant genes in *V. cholerae* strains of the O1 sero-group El Tor biovar as determined by PCR

Strain identification	Place, year and source of isolation	The presence of the <i>ctxA</i> gene	Sensitivity to AMD				Presence of drug-resistant genes				
			Tc	Cip	Cm	Tp	<i>rfbE</i>	<i>tetR</i>	<i>qnrVC</i> (<i>qnrVCI</i>)	<i>floR</i>	<i>dfrA1</i>
ATCC14033	Sinai, 1910, human	—	S	S	S	S	+	—	—	—	—
MAK757	Celebes Island, 1937, human	+	S	S	S	S	+	—	—	—	—
CW6	India, 1966, water	+	S	S	S	S	+	—	—	—	—
34 Qayum	Afghanistan, 1966, human	—	S	S	S	S	+	—	—	—	—
M818, M886, M1051	RF, Astrakhan, 1970, human	+	S	S	S	S	+	—	—	—	—
M642	RF, Astrakhan, 1975, human	+	S	S	S	S	+	—	—	—	—
M1394	RF, Kalmykia, 1979, water	—	S	S	S	S	+	—	—	—	—
M1335, M1397, M1398	RF, Astrakhan, 1981, water	—	S	S	S	S	+	—	—	—	—
M1399, M1400	RF, Astrakhan, 1982, water	—	S	R	S	S	+	—	+	—	—
M1401	RF, Astrakhan, 1982, water	—	S	S	S	S	+	—	—	—	—
25, 33, 95, 113, 121, 317	Guinea, 1986, human	+	S	S	S	S	+	—	—	—	—
99, 190	Guinea, 1986, water	+	S	S	S	S	+	—	—	—	—
M1402	RF, Astrakhan, 1988, water	—	S	S	S	S	+	—	—	—	—
M1264, M1272, M1298, M1299	RF, Krasnodar, 1993, human	+	R	S	R	R	+	+	—	+	+
M1270, M1271	RF, Kazan, 1993, human	+	R	S	R	R	+	+	—	+	+
M1275, M1278, M1279, M1297	RF, Dagestan, 1993, human	+	R	S	R	R	+	+	—	+	+
M1295, M1293	RF, Dagestan, 1994, human	+	S	S	S	R	+	—	—	—	+
M1286, M1287, M1288,	RF, Dagestan, 1994, human	+	S	S	R	R	+	—	—	+	+
M1269	RF, Magnitogorsk, 1994, human	+	R	S	R	R	+	+	—	+	+
P17644	Russia, Achinsk, 1997, human	+	S	S	R	R	+	—	—	+	+
M1318, M1329, M1330, M1336	RF, Astrakhan, 1988–2000, water	—	S	S	S	S	+	—	—	—	—
M1384, M1385, M1386	RF, Astrakhan, 2001, water	—	S	S	S	S	+	—	—	—	—
M1427	RF, Astrakhan, 2003, water	—	S	S	S	S	+	—	—	—	—
M1429	RF, Bashkiria, 2004, human	+	S	S	R	R	+	—	—	+	+

Table 1. (Contd.)

Strain identification	Place, year and source of isolation	The presence of the <i>ctxA</i> gene	Sensitivity to AMD				Presence of drug-resistant genes				
			Tc	Cip	Cm	Tp	<i>rfbE</i>	<i>tetR</i>	<i>qnrVC</i> (<i>qnrVC1</i>)	<i>floR</i>	<i>dfrA1</i>
P18778, P18793, P18796, P18806	RF, Rostov-on-Don, 2005, human	—	S	S	S	S	+	—	—	—	—
P18899	RF, Murmansk, 2006, human	+	S	S	R	R	+	—	—	+	+
L3226	RF, Moscow, 2010, human	+	S	S	R	R	+	—	—	+	+
M1501	RF, Kalmykia, 2011, human	—	S	R	S	S	+	—	+	—	—
M1518	RF, Kalmykia, 2012, water	—	S	R	S	S	+	—	+	—	—
M1524	RF, Kalmykia, 2013, water	—	S	R	S	S	+	—	+	—	—
M1522	RF, Kalmykia, 2014, water	—	S	S	S	S	+	—	—	—	—

All nontoxigenic strains isolated from both the environment and patients in 1998–2014 were sensitive to the studied AMD. However, in Kalmykia in 2011–2013, strains resistant to second-generation fluoroquinolone—ciprofloxacin were isolated (see Table 1).

Selection of DNA Target Sequences and the Development of an Amplification Protocol

Several mechanisms of the formation of drug resistance in bacteria have now been described. Such structures as plasmids, insertion sequences (IS elements), transposons, and integrins with gene cassettes are involved in the distribution of drug-resistant genes. An important role belongs to other complex organized structures—integrative and conjugative elements (ICEs) and integrative mobilized elements (IMEs) and genomic islands. The causative agent of cholera constantly acquires drug-resistant genes via the introduction of mobile genetic elements. In its genome, plasmids, first and second class integrins, as well as SXT elements (Sulfamethoxazole-Trimethoprim), were detected [14, 15]. In this case, the genes determining resistance to a particular drug can often be part of different genetic elements. For example, the *dfrA* (*dfrA1*) gene, which determines the resistance to trimethoprim/sulfamethoxazole, was detected in the SXT element, IncC plasmids, and integrins of the first and second classes [16, 17]. A cassette with *qnrVC* genes providing resistance to ciprofloxacin can be localized both on plasmids and on SXT element [18, 19].

An adequate choice of cholera treatment can only be made based on a preliminary determination of the sensitivity of isolated cholera strains to AMD. We

designed a set of primers and probes for real-time PCR for the simultaneous detection of *V. cholerae* strains and the determination of drug-resistant genes in their genome. With the use of the real-time regime, we planned to reduce the time to determine drug-resistant genes to 3–4 h, simultaneously identifying all four resistant genes. For comparison, analysis by the disc method, E-test method, and dilution methods in a liquid nutrient medium or in agar requires 18–48 h, and the use of singleplex PCR test systems with electrophoretic detection of resistance to only one antibiotic requires 5–8 h [20]. It should be noted that the probability of false positive results in our case is minimized (nucleotide sequences of more than 100 strains of *V. cholerae* were analyzed for the design of primers and probes, and specific primers and probes, as well as PCR conditions, were selected). In addition, we reduced the possibility of contamination of the PCR mixture by excluding the electrophoretic DNA separation step from the determination.

The site of the *rfbE* gene (*vc0244*) from the *rfb* cluster, which encodes the biosynthesis of O1-antigen [21], was selected as the target for pathogen detection. This made it possible to detect *V. cholerae* strains of the O1 serogroup. We decided not identify the bacillus of the O139 serogroup, which caused cholera outbreaks in 1992–1993, since strains of this serogroup are not imported into Russia; they cause local outbreaks only in India.

To determine the resistance of strains to drugs, AMDs are most often used to treat cholera; namely, tetracycline, trimethoprim, chloramphenicol (levomycesin), and ciprofloxacin were chosen. the *tetR* gene was chosen as the DNA target encoding tetracycline resistance, the site of the *floR* gene was selected to detect

Table 2. Primers and hydrolysis probes constructed to detect drug-resistant genes

Primer, probe	Sequence 5'→3'	Detection channel
<i>tetR-F</i>	TCAGTGATAAAGTGTCAGCA	Red
<i>tetR-R</i>	AGTTTGCGTGTCTCGTCAG	
<i>tetR-probe</i>	(Cy5)-TTGCAGCCGAATACAGTGATCCGT-(BHQ2)	
<i>qnrVC1-F</i>	CAAACCTCCGCGATACACAA	Yellow
<i>qnrVC1-R</i>	CGCATTCTCTGAACCTCGATACC	
<i>qnrVC1-probe</i>	(R6G)-TGTAGACTGTTCTTTTCATTGAACGAGGTGA-(BHQ1)	
<i>dfrA1-F</i>	GAATGGAGTTATCGGGAATGG	Orange
<i>dfrA1-R</i>	TCTTGCGTCCAACCAACA	
<i>dfrA1-probe</i>	(ROX)-GGAGTGCCAAAGGTGAACAGCT-(BHQ2)	
<i>floR-F</i>	GGGTTTACACTGTCGGCTTTA	Crimson
<i>floR-R</i>	TTCCGCTTGGCCTATGA	
<i>floR-probe</i>	(Cy5,5)-CGGTATGGGCACCTTCTTCGTCTT-(RTQ2)	
<i>rfbE-F</i>	ACTTATGTTGCCTCGGTTAATA	Green
<i>rfbE-R</i>	GTAGAGACTCACCTTCGATTTC	
<i>rfbE-probe</i>	(FAM)- ATAGTCCAGTGTGGTGCGTTACCC-(BHQ1)	

chloramphenicol resistance, *dfrA1* was chosen to detect trimethoprim resistance, and *qnrVC* (*qnrVC1*) was used to detect fluoroquinolones resistance.

A pair of primers and a hydrolysis probe were calculated for each gene with the IDPrimerQuest online program. During the selection of primers and probes, special attention was paid to their compliance with the requirements for the design of oligonucleotides for TaqMan® systems. In total, five pairs of primers and probes with detection on five different channels were calculated (Table 2).

The PCR mix for the developed amplification variant contained the following components: 8 pmol/μL of each primer, 4 pmol/μL probe; 1.5 units. Taq polymerase; 2.5 μL of 10-fold PCR buffer (pH 8.4), 25 mM MgCl₂ solution, 2 mM dNTP (all components produced by Synthol, Russia); and 5 μL DNA sample and deionized water up to a final volume of 25 μL.

The amplification protocol included the following steps: 1 cycle at 95°C for 5 min; 35 cycles at 95°C for 15 s and 60°C for 60 s with the detection of a fluorescent signal during the annealing stage simultaneously on five channels in one tube. Table 2 shows that the amplification product was detected on an individual channel for each gene: *tetR*—Red channel, *dfrA* (*dfrA1*)—Orange, *floR*—Crimson, *qnrVC* (*qnrVC1*)—Yellow, and *rfbE*—Green channel.

Thus, the proposed method was designed for the simultaneous detection of *V. cholerae* strains of the O1 serogroup and for the identification of therapeutically important resistant genes in their genome by real-time PCR. The developed method uses five sets of specific primers and probes.

Determination of Specificity and Effectiveness of the Developed Primers and Probes Sets

Five toxigenic *V. cholerae* strains of the O1 serogroup classical biovar were used (M29, 16002B, Dacca 3, Dacca 35, B1307), *V. cholerae* O139 (P16064), two *V. cholerae* strains neO1/neO139 (1332-69, 13030), two *E. coli* strains (M17 and 6), *K. pneumoniae* strain 1, *Shigella sonnei* (ATCC 25931), *Salmonella typhi* (ATCC H901), *Salmonella typhimurium* (ATCC 14028), and *Salmonella enteritidis* (WHO) were used to assess the specificity of primers and probes (Table 3). It is known that the classical biovar strains do not contain drug-resistant genes [15]. Indeed, only the *rfbE* gene was detected in these strains, and all other tested drug-resistant genes were absent (Table 3).

At the same time, negative PCR results were obtained for *V. cholerae* strains of neO1/neO139 serogroup, *E. coli*, *K. pneumoniae*, *Salmonella enteritidis*, *S. typhimurium*, *S. typhi*, and *Shigella sonnei* for the determination of the site of the *rfbE* gene. These results confirm the specificity of the selected primers and probes. *V. cholerae* strain O139 contained only the *floR* gene. The obtained data correspond to the literature data, according to which the toxigenic strains of the O139 serogroup contain the SXT element, which simultaneously determines resistance to four AMDs: sulfamethoxazole (*sulII*), trimethoprim (*dfr18*), chloramphenicol (*floR*), and streptomycin (*strB*) [22]. However, the trimethoprim-resistant gene in strains of the O139 serogroup (*dfr18*) had a structure different from the *dfrA1* gene of the El Tor bacillus, and this allele was not detected by our system.

Table 3. Specificity of the developed PCR

Strain	Place, year and source of strain isolation	Presence of drug-resistant genes				
		<i>rfbE</i>	<i>tetR</i>	<i>qnrVC</i> (<i>qnrVCI</i>)	<i>floR</i>	<i>dfrA1</i>
<i>V. cholerae</i> O1 M29	RF, Astrakhan, 1942, human	+	—	—	—	—
<i>V. cholerae</i> O1 16002B	India, 1944, human	+	—	—	—	—
<i>V. cholerae</i> O1 Dacca 3, Dacca 35	Pakistan, 1958, human	+	—	—	—	—
<i>V. cholerae</i> O1 B1307	Pakistan, 1964, human	+	—	—	—	—
<i>V. cholerae</i> O139 P16064	RF, Azov, 1993, human	—	—	—	+	—
<i>V. cholerae</i> nonO1/nonO139 1332-69	Sudan, 1967, u/k	—	—	—	—	—
<i>V. cholerae</i> nonO1/nonO139 13030	RF, Astrakhan, 1976, u/k	—	—	—	—	—
<i>Salmonella typhi</i> ATCC H901	RF, 1918, u/k	—	—	—	—	—
<i>S. enteritidis</i> WHO	U/k, 1956, u/k	—	—	—	—	—
<i>S. typhimurium</i> ATCC 14028	Unknown	—	—	—	—	—
<i>E. coli</i> M17	RF, Saratov, u/k, water	—	—	—	—	—
<i>E. coli</i> 6	RF, Saratov, 2016, human	—	+	—	—	—
<i>K. pneumoniae</i> 1	RF, Saratov, 2016, human	—	+	—	—	+
<i>Shigella sonnei</i> ATCC 25931	Panama, u/k, human	—	—	—	—	—

U/k, source is unknown. Designations of drug-resistant genes are given in Table 1.

The regions of the *floR* and *qnrVC* (*qnrVCI*) genes in the used *V. cholerae* strains of neO1/neO139 serogroup, *E. coli*, *K. pneumoniae*, *Salmonella enteritidis*, *S. typhimurium*, *S. typhi*, and *Shigella sonnei* were not detected. However, *tetR* and *dfrA1* genes were detected in the genome of the *K. pneumoniae* 1 strain, and the *tetR* gene was identified in the genome of the *E. coli* strain 6 (Table 3). First, the obtained data indicated a wide distribution of tetracycline- and trimethoprim-resistant genes among various microorganism groups. Second, they demonstrated that the designed primers and probes for the detection of regions of the *tetR* and *dfrA1* genes can be used to detect these genes, not only in *V. cholerae* but also in other microorganisms.

The sensitivity of the analysis of the pure culture of *V. cholerae* strains with a constructed panel was 1×10^3 CFU/mL.

The developed kit was later tested with 60 natural *V. cholerae* strains of the O1 serogroup El Tor biovar (Table 1). Among them, two strains were related to prepanemics (ATCC 14033 and MAK 757), 12 were related to toxigenic typical strains isolated at the beginning and initial years of the current pandemic (1966–1986), and 20 were related to toxigenic strains of atypical El Tor strains imported in 1993–2010. Another 26 strains isolated from both humans and water from environmental reservoirs were atoxigenic (Table 1).

Clinical *V. cholerae* strains of the O1 serogroup El Tor biovar isolated before 1993 did not carry the *floR*, *dfrA1*, *qnrVC* (*qnrVCI*), or *tetR* genes (see Table 1). The obtained PCR results correspond to the data from

the disc diffusion method and the literature data showing high sensitivity of the prepanemic and clinical *V. cholerae* O1 biovar El Tor, isolated during this period of the current pandemic [11, 23] to the AMD. At the same time, 88% (14 of 16) of the clinical *V. cholerae* strains of biovar El Tor isolated after 1993 carried *floR* and *dfrA1* genes (possibly due to the presence of the SXT element in the genome). Figure 1 shows the results of the determination of the *floR* gene in some typical and atypical El Tor strains. It can be seen that this gene was found in strains M1264, M1279, and L3226 (signal above the threshold), and it was not detected in strains M818, 113, M1293, P18796, or M1501 (signal below the threshold).

In 11 of the 16 strains isolated after 1993, the *tetR* gene was also detected with the developed PCR system (see Table 1). However, atypical El Tor strains isolated during the modern period (2004–2010) did not carry this gene.

All nontoxigenic strains were deprived of the *floR*, *dfrA1*, and *tetR* genes. At the same time, the *qnrVC* (*qnrVCI*) gene, which is responsible for ciprofloxacin resistance, was detected in the genome of two strains isolated from river water in Astrakhan in 1982. This gene was also detected in one clinical (2011) and two aquatic (2012–2013) strains isolated in the modern period in Kalmykia (Table 1).

In addition, we studied the use of a constructed kit to analyze samples contaminated by the cholera pathogen from the environment (river water, food products) and samples simulating clinical material (1% liquid potato starch). All samples, including environmental

Fig 1. Accumulation of the fluorescent signal of amplified product (*floR* gene) in *V. cholerae* strains (detected by Crimson channel): 1—M1264, 2—M1279, 3—L3226, 4—M818, 5—113, 6—M1293, 7—P18796, 8—M1501.

samples, were sterilized by autoclaving at $126 \pm 2^\circ\text{C}$. The samples contained *V. cholerae* suspensions in a concentration from 1×10^9 to 1×10^2 CFU/mL. As a result, it was found that the kit sensitivity for these samples was 1×10^3 CFU/mL (data not shown). Taking into account that the clinical material and environmental samples could be contaminated with other microorganisms containing AMD-resistant genes, the proposed variant of PCR should be performed only for colonies that may contain cholera bacilli.

CONCLUSIONS

Thus, a multiplex real-time PCR method was developed for the simultaneous detection of *V. cholerae* strains of the O1 serogroup and the determination of the presence of resistant genes to four drugs used to treat cholera (chloramphenicol, trimethoprim, tetracycline, and ciprofloxacin) in their genomes. The sensitivity of the constructed set of primers and probes is 1×10^3 CFU/mL. The effectiveness of the developed method was confirmed via the analysis of 60 natural *V. cholerae* strains isolated over different years from people and from the environment.

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